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Effect of Increasing Dose of Interferon on the Evolution of Hepatitis C Virus 1b Quasispecies

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The effects of interferon therapy on hepatitis C virus (HCV) genome are still controversial in terms of biological and clinical significance. Changes in the quasispecies of the hypervariable (HVR) and non-structural 5A (NS5A) regions of HCV 1b were evaluated in nine patients treated with increasing doses of interferon and five untreated controls. HCV quasispecies were analyzed in HVR and NS5A by single-strand conformation polymorphism assay. The HVR quasispecies varied over time both in treated and untreated patients. However, at least one persistent strain was present in all patients. With low doses of interferon, variations in HVR complexity were found in seven of nine patients and in four patients new variants became detectable. A reduction in the heterogeneity of the HVR quasispecies was observed after increase of the interferon dose. In contrast, NS5A profiles remained unmodified in all but three cases in which direct sequencing showed no changes in amino acid sequences of the predominant strain. The results suggest that interferon sensitivity of some HCV strains may be dose dependent. The homogeneity of NS5A pattern populations during treatment suggests that interferon exerts much less pressure on this region. ***J. Med. Virol. 60:133–138, 2000.***

KEY WORDS: hepatitis C virus; hypervariable region 1; non-structural 5A region; single-strand conformation polymorphism

INTRODUCTION

Hepatitis C virus (HCV) is the major etiological agent of chronic hepatitis, a common disease with variable clinical course that may lead to cirrhosis and hepatocellular carcinoma [Houghton et al., 1991; Tong et al., 1995; Yano et al., 1996]. The mechanisms of viral persistence are still undefined, but chronicity of HCV infection develops despite a vigorous humoral and cellular response to several structural and non-structural viral proteins [Koziel et al., 1993].

HCV genome, like most RNA viruses, consists of a heterogeneous population of highly related but genetically distinct variants referred to as quasispecies [Domingo et al., 1985; Martell et al., 1992]. Such a genomic variability, especially in the E2 envelope protein encoding sequences of the hypervariable region (HVR1), could enable the virus to escape host immune surveillance. Thus, genomic heterogeneity may have a major role among the mechanisms influencing the persistence of infection and the outcome of antiviral therapy with alpha-interferon (IFN) [Bukh et al., 1995; Gonzalez-Peralta et al., 1996].

In addition to pre-treatment high viral load and genotype 1b, a high degree of viral genome complexity, as determined by the number of quasispecies populations, seems to correlate with a lower rate of response to treatment [Kanazawa et al., 1994; Martinot-Peignoux et al., 1995; Moribe et al., 1995; Pawlotsky et al., 1998], although a recent study of a large number of patients suggested that the degree of HCV heterogeneity was not related to the type of response to IFN therapy [Lopez-Labrador et al., 1999]. The mechanism of action of IFN in HCV infection is known only partially, but its immunomodulatory, anti-inflammatory, and antiviral properties may play an important role [Tilg, 1997]. Recently, an interferon sensitivity determining region (ISDR) within the non-structural 5A (NS5A) domain of HCV genome has been identified [Enomoto et al., 1995]. Multiple mutations within the ISDR, likely resulting in amino acid sequence changes, have been shown to favour the response to IFN therapy in Japanese patients with HCV genotype 1b infection [Eno-

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TABLE I. Clinical, Biochemical, and Histological Characteristics of HCV 1b Non-responder Patients to IFN Treatment and Control Group*

Pts[a]	Sex	Age (years)	ALT-B (IU)[b]	ALT-1 (IU)[c]	ALT-2 (IU)[d]	Source of infections	Months of disease	Histology CAH
P1	M	57	58	59	45	Unknown	72	Mild
P2	F	42	99	68	57	Unknown	48	Mild
P3	M	51	165	220	47	Unknown	24	Moderate
P4	F	48	122	130	125	Unknown	60	Moderate
P5	F	55	141	130	92	Unknown	36	Mild
P6	M	53	55	81	61	Unknown	40	Mild
P7	F	55	70	61	45	Unknown	9	Moderate
P8	M	47	167	132	176	Unknown	36	Moderate
P9	F	55	348	120	50	Unknown	24	Mild
C1	F	35	48	55	45	Unknown	12	Mild
C2	F	53	120	169	179	Parenteral	120	Moderate
C3	M	47	208	50	48	Unknown	24	Mild
C4	F	41	62	45	69	Unknown	24	Mild
C5	F	45	81	137	136	Unknown	48	Moderate

*HCV, hepatitis C virus; ALT, alanine aminotransferase; CAH, chronic active hepatitis.
[a]Treated patients (P#) and controls (C#).
[b,c,d]Serum ALT values at intervals of 4 months: ALT-B, pretreatment values; ALT-1 and ALT-2, during therapy at 3MU and 6MU, respectively. (ALT normal range: <40 IU.)

moto et al., 1996]. These findings, however, have not been confirmed in European and North American patients [Khorsi et al., 1997; Zeuzem et al., 1997; Polyak et al., 1998]. Finally, it is still not clear whether the determinants of IFN resistance are, at least in part, dose dependent [Carithers and Emerson, 1997].

In the present study, the behaviour of HCV populations under IFN therapy was evaluated to assess whether: IFN-resistant HCV populations or viral strains are selected in the course of therapy; and whether this phenomenon is related to the dosage of IFN employed. Single-strand conformation polymorphism analysis (SSCP) was used to study the evolution of HVR1 and NS5A genomic profiles in patients treated unsuccessfully with increasing doses of IFN.

MATERIALS AND METHODS

Patients

Nine patients with chronic hepatitis C, non-responders while treated with increasing doses of IFN, were studied. Non-response was defined on the basis of biochemical and virological results as the absence of any significant variation of alanine aminotransferase (ALT) levels and persistence of HCV RNA in serum at all time points evaluated during treatment. All patients (four men and five women) aged 42–57 years, had abnormal ALT levels for at least 6 months, with serum HCV RNA before therapy. The infecting HCV was genotype 1b, according to Simmonds' classification [Simmonds et al., 1994]. Pre-treatment histological examination showed mild chronic hepatitis in five cases and moderate chronic hepatitis in the other four. In all patients, the IFN dose was increased from the standard dose of 3 MU to 6 MU three times weekly, if a biochemical and virological response had not occurred after 4 months. During the 8 months of treatment, ALT levels were monitored monthly, and the presence of serum HCV RNA by nested polymerase chain reaction (PCR) on 5′-untranslated region (UTR) was investigated before therapy (B), after 4 months of treatment at the dose of 3 MU (time point 1), and after a further 4 months at 6 MU (time point 2). In addition, three sequential samples taken at intervals of 4 months were collected from five control patients with chronic hepatitis C (infecting genotype 1b) who did not receive IFN therapy. Clinical and histological findings of treated and untreated patients are summarised in Table I.

HCV populations patterns were analyzed on HVR and the NS5A region by SSCP assay before treatment and during escalating doses of IFN therapy.

RNA Extraction

RNA was extracted from serum with single-step acid guanidinium thiocyanate-phenol-chloroform method [Chomczynsky and Sacchi, 1987]. Briefly, 200 μl of serum were mixed with 800 μl of guanidinium buffer, 2 mol/l sodium acetate, 800 μl of diethylpirocarbonate water-saturated phenol and 150 μl of chloroform-isoamilic alcohol. The extracted RNA was dissolved in 10 μl of sterile diethylpirocarbonate-treated water and 10 U of RNase inhibitor (Pharmacia, Uppsala, Sweden).

5′-UTR-Nested PCR

Five microliters of a reverse transcription mixture were adjusted to contain 2 μl of extracted RNA, 50 U of Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) and its buffer (BRL Life Technologies, Gaithersburg, MD), 10 U of RNase inhibitor, 5 pmol of downstream primer, 1 μmol/l of each deoxynucleotide triphosphate (dNTP), and 10 mmol/l of dithiothreitol. After incubation at 37°C, the complementary DNA (cDNA) was amplified in 100 μl of PCR mixture with 10 pmol of outer primer set encompassing 5′-UTR of HCV genome, for 30 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 1 min). The nested-PCR was performed with 10 pmol of each inner primer and 2 μl of the first amplification products as template, with the same cycle conditions

[De Mitri et al., 1995]. The recommended precautions were taken to reduce the risk of contamination of samples with PCR products, and positive and negative samples were included in each series [Kwok and Higuchi, 1989].

HCV Genotyping

The genotypes and subtypes of infecting HCV were carried out on 5′-UTR using a reverse hybridisation assay (InnoLiPA, Innogenetics, Zwijndrecht; Belgium) [Stuyver et al., 1993] in which the amplification products were hybridised to oligonucleotide probes specific for the different HCV genotypes.

HVR1 and NS5A Nested Asymmetric PCR

cDNA solution obtained with downstream outer primer on the hypervariable [Koizumi et al., 1995] and non-structural 5A [Zeuzem et al., 1997] regions was made up to 25 μl of a PCR mixture containing outer primer set designed from HVR1 and NS5A of HCV gene. Forty cycles of PCR were performed with the difference of a higher annealing temperature (60°C) for the HVR amplification. To generate single-stranded DNA, 2 μl of PCR products were used for nested asymmetric PCR with downstream outer and upstream inner primers present in different molar concentrations (1:10), so that when the primer present in limiting amounts had been used up, an excess of single-stranded DNA was produced [De Mitri et al., 1998].

An aliquot of the 5′-UTR, HVR1, and NS5A amplification products was analyzed by electrophoresis on 1.5% agarose gel stained with ethidium bromide and visualised under ultraviolet light.

Non-Isotopic SSCP

SSCP analysis was carried out as described previously [De Mitri et al., 1998]. Briefly, single-stranded DNA amplification products were denatured and subjected to electrophoresis on modified non-denaturing acrylamide gel (MDE gel, AT Biochem, Malvern, PA). The bands were highlighted by silver staining (Bio-Rad, Hercules, CA). For the genomic three-dimensional conformation, denatured single stranded DNA can be separated into different bands derived from viral quasispecies, in non-denaturing conditions [Orita et al., 1989].

Direct Sequencing of PCR NS5A Product

The amplification products derived from the NS5A region were sequenced directly by means of an ABI 373 automated sequencer using PRISM dye terminator cycle sequencing (Perkin-Elmer, Foster City, CA) according to the manufacturer's instructions. Both upstream and downstream strands were sequenced for each PCR sample. HCV sequences were analyzed using the Sequencer 3.0 analysis program (Gene Codes, Ann-Arbor, MI). Deduced amino acid alignment was performed using Clustal W computer program [Thompson et al., 1994].

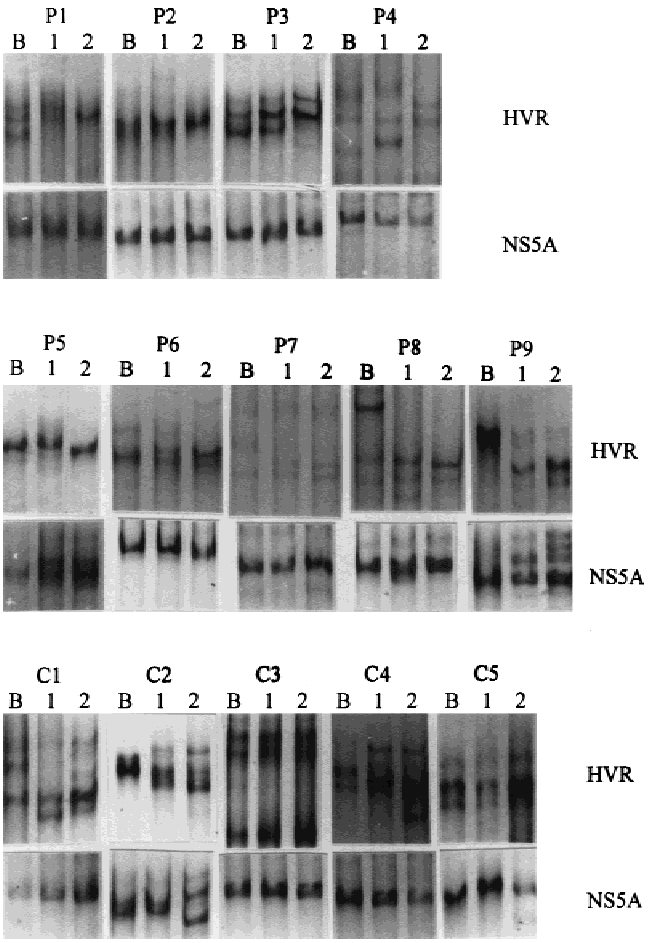

Fig. 1. Polymerase chain reaction-strand conformation polymorphism (PCR-SSCP) profile on hypervariable (HVR) and non-structural 5A (NS5A) regions of hepatitis C virus (HCV) genome in nine non-responder patients to escalating doses of alpha-interferon (IFN) therapy and five untreated patients. In treated patients (P#), the SSCP analysis was performed before treatment (time point B), after 4 months at the standard dose of 3MU (time point 1) and after another 4 months at 6MU (time point 2) of IFN treatment. In untreated patients (C#), the SSCP pattern was evaluated in sequential samples collected at intervals of 4 months (B, 1, 2).

RESULTS

The profiles of HVR1 and NS5A quasispecies during the observation period in treated and untreated patients are shown in Figure 1. Baseline HVR quasispecies profile showed multiple HCV variants in all but one patient (P5) and in all the controls. Changes in SSCP patterns were observed during IFN treatment, but one or more strains detected before treatment persisted in all three samples of each patient. Only one patient (P5) had identical quasispecies profiles in the three time points analyzed. At the first time point, after 4 months of therapy with a low IFN dose (3 MU), changes in HCV complexity had occurred in seven of the remaining eight patients with the disappearance of pre-existing bands in three cases (P1, P4, P8) and the emergence of new bands in the other four patients (P2, P6, P7, P9). In three of the latter patients (P6, P7, P9), the new strains also persisted after the 4-month treat-

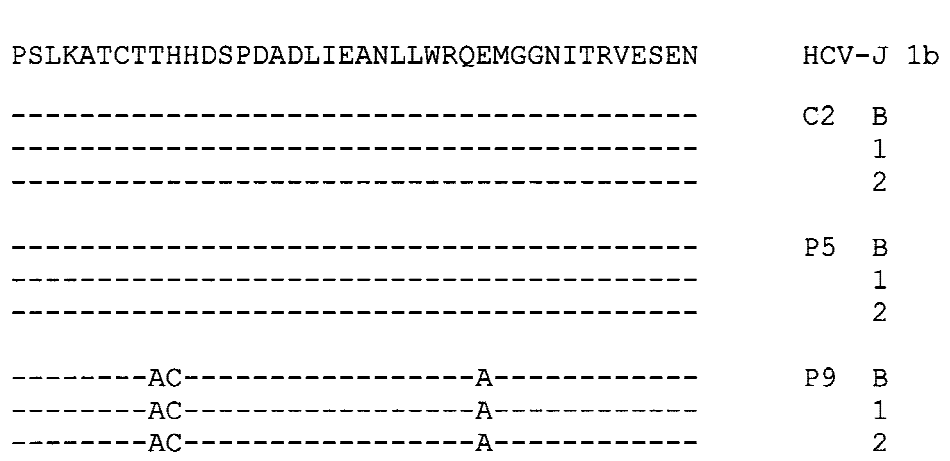

Fig. 2. Amino acid sequences in non-structural 5A (NS5A) region, encompassing an interferon sensitivity determining region (ISDR) ($NS5A_{2209–2248}$), in two patients (P5, P9) and one control (C2) who showed different hepatitis C virus (HCV) quasispecies profiles on strand conformation polymorphism (SSCP) assay. The sequence was performed on three time points at intervals of 4 months corresponding to pre-treatment (B), and during escalating doses of alpha-interferon (IFN) (3MU, time point 1; 6MU, time point 2). The NS5A amino acid sequence of HCV-J 1b is shown in capital letters at the top. Dashes indicate identity with the prototype sequence; amino acid substitutions are indicated.

ment with 6 MU of IFN. In the other five patients (P1, P2, P3, P4, P8), a reduction of HCV populations was observed after the treatment with 6 MU. Variations in the HVR1 quasispecies were found also in all but one untreated patient (C3), and the changes of quasispecies distribution showed an increased complexity in the sequential time points examined.

The SSCP analysis of the NS5A gene, encompassing the ISDR, showed a less heterogeneous pattern than HVR1.

A single predominant strain was detected in seven patients and in four controls, whereas different SSCP profiles appeared in the sequential samples of the remaining three cases (two treated and one untreated). In particular, two treated patients (P5, P9) had a single strain in the pre-treatment samples, whereas the SSCP patterns during the increasing dose of IFN showed a more complex population. This was characterised by an additional strain in both samples, in one case (P5), whereas in the other (P9), two and three additional strains became detectable at the first and second time points, respectively. The untreated patient (C2) showed the same SSCP pattern in two samples, whereas it changed in the last sample. In these three patients (P5, P9, C2), mutations in the prevalent HCV strain were searched for by direct sequencing of the NS5A amplification products (Fig. 2). Only sporadic silent mutations in the nucleotide sequence were found at all time points examined. When deduced amino acid sequences of putative ISDR (position 2209–2248) were compared with HCV-1b prototype sequences (HCV-J) [Kato et al., 1990], amino acid changes in three positions (2217, 2218, 2236) were found in one patient only (P9), as shown in Figure 2. Following the Enomoto criteria [Enomoto et al., 1996], this patient proved to be infected by the intermediate strain whereas the other two cases (P5, C2) were infected by wild-type HCV. By comparing the SSCP profiles in HVR and ISDR in these three patients, no relationship in the HCV quasispecies profiles were found.

DISCUSSION

More than half of HCV-infected patients do not respond to treatment with alpha-interferon. Recent studies have suggested that genomic characteristics of HCV may play a role in resistance to IFN therapy. An association between high genetic variability within the HVR1 and treatment failure has been repeatedly described, while controversial results have been reported about a possible relationship between the NS5A sequence and response to IFN therapy [Khorsi et al., 1997; Zeuzem et al., 1997; Polyak et al., 1998].

The effect of IFN therapy on the evolution of the HVR and NS5A (ISDR) quasispecies was assessed in patients infected by HCV 1b, non-responders to different, increasing doses of IFN. In agreement with previous reports [Kurosaki et al., 1993; Enomoto et al., 1994], a significant time-related evolution of the HVR quasispecies was observed in most treated and untreated patients, who already had a rather high degree of quasispecies complexity at baseline. The present study also allowed us to evaluate the effect of different, increasing doses of IFN on genomic HCV variability.

During the administration of the 3MU dose of IFN, some pre-existing strains disappeared in three cases and further HCV variants were cleared in five patients when the IFN dose was increased. This finding suggests that the sensitivity to IFN of these strains was dose dependent. However, the new variants appeared in three patients during the treatment with 3MU of IFN, persisting during the 6MU treatment. All patients showed the persistence of one or more pre-existing strains throughout the entire treatment period, indicating that dose escalation was not effective on these strains of HCV.

A time-related evolution of the HVR1 quasispecies was also observed in the untreated patients, but its pattern was strikingly different from that of treated patients, as most showed a HVR1 complexity increasing with the time.

Enomoto et al. [1995] identified recently a small sequence in the carboxy-terminal region of NS5A gene

(ISDR) that seems to determine the HCV sensitivity to IFN. Patients carrying HCV 1b with wild-type ISDR or with 1–3 amino acid substitutions (intermediate type) usually failed to develop a sustained response to therapy, whereas multiple substitutions in this amino acid sequence were associated with sustained clearance of the virus [Enomoto et al., 1996]. The potential importance of the link between NS5A region and sensitivity to IFN has been reinforced by a study showing that ISDR modulates the response to therapy through the inhibition of protein kinase R, a cellular protein induced by IFN [Gale et al., 1997]. In the present study the mutations in ISDR were not examined in relation to the response to IFN treatment, but the evolution of viral NS5A patterns during IFN therapy administered at increasing doses was studied. The analysis of SSCP profiles showed that most patients, both treated and untreated, only harboured one strain that persisted throughout the observation period. In three patients (two treated and one untreated), new strains appeared over time. However, the comparison of the nucleotide sequences between each time point revealed only silent nucleotide mutations with no amino acid change. The discrepancy between the results obtained by direct sequencing and SSCP assay may be due to the different sensitivity of the two techniques. Indeed, direct sequencing of amplification products is able to detect mutations only within the predominant strain and the presence of co-existing minor variants may be overlooked. The comparison of the amino acid sequences in these three patients with the prototype HCV-J 1b showed that infecting HCV was wild type in two cases and intermediate type in the other. There are controversial issues concerning the evolution of NS5A populations during IFN treatment. In particular, Zeuzem et al. [1997] and Polyak et al. [1998] have shown that IFN therapy exerts pressure on the ISDR, even though this finding was limited to a subset of patients. In contrast, but in agreement with Squadrito et al. [1997], we did not find significant mutations in the NS5A sequence during IFN treatment. The present study also showed that even with increasing doses of IFN, no changes in HCV populations occurred in the NS5A region, and none of the variant strains selected during therapy became predominant in quasispecies populations.

In conclusion, it was found that fluctuations of HCV quasispecies in HVR1 can be detected both in treated and untreated patients infected with HCV 1b, and the evolution of some HCV strains is likely related to intrinsic or immune-mediated heterogeneity of HVR rather than to a selective pressure induced by IFN. In fact, resistant strains may be selected before treatment and the resistance of these variants is not related to the dosage regimen. On the other hand, the reduction of HCV populations in HVR1 with a more aggressive treatment suggests that the sensitivity to IFN may be dose dependent in some strains. Thus, higher doses or daily administration of IFN may be more effective in reducing the complexity of HCV quasispecies pattern.

Finally, unlike HVR findings, the NS5A region is more homogeneous genetically in the majority of both treated and untreated patients. Sequencing of ISDR did not show any significant change in nucleotide sequences of the predominant infecting strain in patients showing a heterogeneous SSCP pattern.

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